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EFFECT OF THE ANTICOAGULANT FRACTION OF INCUBATED
FIBRINOGEN ON PROTHROMBIN CONSUMPTION

BY

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A THESIS

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "EFFECT OF THE ANTICOAGULANT FRACTION OF INCUBATED FIBRINOGEN ON PROTHROMBIN CONSUMPTION", submitted by CHIN-CHI CHEN in partial fulfilment of the requirements for the degree of Master of Science.

ABSTRACT

A study was undertaken to investigate further the effect of AFIF (Anticoagulant Fraction of Incubated Fibrinogen) on prothrombin consumption using the one-stage method of Quick. The present investigation shows that AFIF itself, not the contaminating fibrinolysin,* inhibits the consumption of prothrombin. This property is unaffected by the length of incubation of the parent fibrinogen solution. The degree of inhibition of the prothrombin consumption depends only on the final concentration of blood and anticoagulant. Experiments with the two main electro-phoretic fractions of AFIF indicate that both of them interfere with normal consumption. Substitution of platelets with hemolysate or rabbit brain phospholipid (Bell - Alton reagent) can abolish the inhibitory effect of AFIF.

* The term fibrinolysin (or plasmin) is used for the enzyme whether it hydrolyzes fibrin or fibrinogen. However, the term fibrinolysis is used when the former activity is referred to, and fibrinogenolysis for the latter.

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I. INTRODUCTION

Fibrinogen, one of the essential proteins required for blood coagulation, has been well known for a long time (1). However, the anticoagulants derived from this substance after incubation were noticed only in recent years. In 1934 Garner and Tillett (2) observed that when human fibrinogen was incubated with streptococcal fibrinolysin*, the former could neither be clotted by thrombin nor be precipitated at 50% sodium chloride saturation or at 25% ammonium sulfate saturation as intact fibrinogen can. Higher sulfate concentrations, i.e. 35%, were required. The incubated fibrinogen also became more heat resistant; however, it did not lose the characteristics of a protein. In 1945 Seegers, Niefert and Vandenbelt (7) showed that when alcohol (7%) precipitated bovine fibrinogen preparations stood at room temperature for several days (approximately 6 days), they underwent the same alterations spontaneously. These investigators attributed these changes to the activation of the fibrinolytic system, which usually contaminates fibrinogen preparations. In the same year (1945), Banfi, Tanturi and Ray (8) found a prolongation of the thrombin time of stored plasma and postulated the formation of an anti-thrombin in this plasma. However, no further details for this antithrombic property were given. Similar phenomena were also observed later by Stefanini (9). However, none of these investigators suggested that this antithrombic activity might have been derived from fibrinogen. To the contrary, Stefanini claimed that the fibrinogen obtained from human plasma

* It was renamed streptokinase by Christensen because the streptococcal product was an activator rather than a fibrinolysin (3, 4, 5, 6).

stored for thirty days was still acted upon normally by thrombin at full strength or at lower concentrations.

In 1954, Ratnoff (10) observed that fresh plasma shortened the thrombin time of stored plasma and assumed that normal plasma contained a heat-labile, non-dialyzable fibrinogen conversion accelerator which accelerated the coagulation of fibrinogen by thrombin. This accelerator disappeared during storage. Triantaphyllopoulos, Quick, and Greenwalt (11) on the other hand noticed that incubation increased the thrombin time of native and oxalated plasma and when this incubated plasma was mixed with fresh plasma it prolonged the thrombin time of the latter. Instead of assuming disappearance of an accelerator, they postulated a heparin-like substance formed during incubation which inhibited the conversion of fibrinogen to fibrin by thrombin. In subsequent experiments, Triantaphyllopoulos (12, 13) showed that plasma and fibrinogen but not serum acquired anticoagulant properties upon incubation and that the substance responsible for these properties was not identical with heparin. Similar observations were reported by Stormorken (14) in Norway and Niewiarowski and Kowalski (15) in Poland. Since the anticoagulant properties were first shown to be associated with the fraction which was precipitated between 25 and 50% ammonium sulfate saturation of incubated fibrinogen (12, 13), the name anticoagulant fraction of incubated fibrinogen (AFIF) was given to this material (16). However, it was found later (17, 18) that once fibrinogenolysis has advanced beyond a certain stage, the fraction precipitated at 25% ammonium sulfate saturation also exhibits antithrombic activity.

It has been shown that AFIF inhibits the clotting of fibrinogen by thrombin in a competitive manner as indicated by a Lineweaver-Burk plot

(16, 19) and that neither thrombin nor fibrinogen are destroyed (13). AFIF interferes with both the hydrolytic action of thrombin on fibrinogen (18, 20, 21, 22, 23, 24) and the polymerization of the fibrin monomer (18, 19, 22, 23, 25, 26). It prevents blood from clotting in vivo (28) as well as in vitro (12, 13, 16, 27). It inhibits the consumption of prothrombin (13), retards the formation of plasma thromboplastin (29, 30) and decreases the concentration of factor V by allowing its consumption (13). The inhibitory effect of AFIF on the clotting of intact fibrinogen has been (13, 17, 23) shown to vary with the stage of incubation of the parent fibrinogen during which the anticoagulant was isolated. The inhibition is low at the beginning of incubation, it reaches a peak when the fibrinogen solution becomes completely unclottable by thrombin (i.e. at the end of the clottable period) and decreases to a low plateau during advanced stages of incubation.

As the experiments of Seegers et al. (7) and those of Triantaphyllopoulos (12, 13) and Stormorken (14) indicated, it was not necessary to add active fibrinolysin (plasmin) to a fibrinogen solution to induce digestion.* Fibrinogen solutions contain varying amounts of profibrinolysin (plasminogen) as a contaminant. This proenzyme becomes spontaneously activated upon incubation or standing and lyses fibrinogen in a period of days or months, depending on the amount of contamination and temperature of storage. This view is further supported by the fact that bovine fibrinogen, which is considered free from profibrinolysin when prepared by the method of Laki (31), does not give rise to an anticoagulant upon incubation. It is also supported by the finding (32) that the electrophoretic patterns of incubated human fibrinogen and fibrinolysed human fibrinogen are very similar.

It has been shown (17, 18, 25, 33) that fibrinogenolysis is a continuous and progressive process. Fibrinolysin digests fibrinogen beyond

* The enzymatic breakdown of fibrin or fibrinogen is described by various authors as digestion, degradation or decomposition.

the point of the loss of its clottability by thrombin (17, 18).

In pioneer work, Seegers et al. (7) separated two main components from decomposed bovine fibrinogen. The major one, alpha-derivative, had electrophoretic properties similar to those of intact fibrinogen and was heat-coagulable at 51°C. The minor component, beta-derivative, had electrophoretic mobility values between those of albumin and alpha-globulin and was not heat coagulable. Similar results were reported later by Alkjaersig et al. (19) who believe that the alpha-derivative is predominantly responsible for the inhibition of fibrin polymerization. Kowalski et al. (34) demonstrated that during the digestion of I¹³¹ - labeled fibrinogen by plasmin, TCA (trichloroacetic acid) - soluble, I¹³¹ - containing, low molecular weight products, were released. At the same time high molecular weight products, with electrophoretic mobilities similar to alpha, beta, and gamma globulins, were formed as well as albumin like faster-moving substances. The remaining "core" after the digestion of the fibrinogen was electrophoretically indistinguishable from the original fibrinogen. By comparison of successive radioelectrophoretograms, they concluded that there existed a possibility of mutual interconversion of the high molecular weight products. Using Chromatography on DEAE-cellulose, Nussenzweig, Seligmann, Pelmont and Grabar (35) were able to separate five components from plasmolyzed human fibrinogen which they used in their ultracentrifugation and diffusion studies. They found that the two major constituents D and E had very different properties. Component D, corresponding to the alpha-derivative of Seegers et al., which represents 50% of the total products, had an electrophoretic mobility between that of the beta and gamma globulins of human serum, a molecular weight of approximately 83,000 and was thermolabile. Component E - (beta-derivative of Seegers et al.) had a mobility similar to that of a fast alpha globulin, a

molecular weight of approximately 35,000 and was thermostable. Similar patterns have been observed in degraded dog fibrinogen by Lewis and Wilson (36) recently. The molecular weight for products D and E (200,000 and 100,000) derived from gel filtration data obtained by these authors differ greatly from those estimated by Nussenzweig et al. Only product D was shown to have antithrombic activity. Using continuous flow paper electrophoresis (17, 30) and column electrophoresis (37), Triantaphyllopoulos and Triantaphyllopoulos detected 5 components (17) in AFIF and were able to separate the two main sub-fractions in quantities sufficient for coagulation studies. Both sub-fractions were shown to inhibit the clotting of fibrinogen. The degree of inhibition exerted by the major electrophoretic fraction or larger peak (similar to alpha-derivative of Seegers et al. and D component of Nussenzweig et al.) is high at the beginning of the unclottable period (when separation into fractions could first be obtained) but decreases considerably during advanced stages (18, 37) of incubation following the same pattern which the original non-electrophoresed sample exhibits (17, 18, 37). The inhibition obtained by the small fraction, or small peak (similar to beta-derivative of Seegers et al. and E component of Nussenzweig et al.) is much lower and changes little with incubation (18, 37). The small peak was shown in addition to be able to retard the generation of the intrinsic prothrombin activator (30). Niewiarowski et al. (29) have reported that a thromboplastin generation inhibitor (TGI) was produced during proteolysis of fibrinogen by plasmin. This substance (TGI) must be identical to the small peak of Triantaphyllopoulos and Triantaphyllopoulos. They are both thermostable (60°C, 20 minutes) and have the same rate of appearance during fibrinogenolysis different from that of the major antithrombic component.

As it was shown earlier (13) the prothrombin of human blood

rendered uncoagulable by the addition of AFIF is not consumed during incubation at 37°C. Since these studies were performed without adding an antiprotease into the blood - AFIF mixture, the argument could be advanced that the prothrombin consumption inhibiting effect of AFIF was not due to an intrinsic property of the anticoagulant but to the contaminating fibrinolysin. Fibrinolysin is known to destroy readily both factor V (Ac-globulin, labile factor) and factor VIII (platelet cofactor I, anti-hemophilic globulin), which are indispensable for the rapid conversion of prothrombin to thrombin.

The objectives of the present study are:

- 1) To find out whether the inhibition of prothrombin consumption is due to contaminating fibrinolysin or to an intrinsic property of AFIF itself.
- 2) To investigate whether the activity of AFIF on prothrombin consumption varies, as does its antithrombic activity, with the stage of incubation of the parent fibrinogen solution.
- 3) To establish whether one or both main electrophoretic components of AFIF are responsible for the inhibition of prothrombin consumption.
- 4) To evaluate the effect of platelet factor 3 substitutes (hemolysate, Bell-Alton reagent) on the inhibitory effect of AFIF on prothrombin consumption.

II. MATERIALS AND METHODS

A). Saline

This is a 0.85% (W/V) solution of sodium chloride.

B). Oxalated Saline

Oxalated saline was prepared by diluting one part of 0.1 M sodium oxalate with nine parts of saline (final concentration of oxalate 0.01 M).

C). Imidazole Buffer

The buffer was prepared as described by Mertz et al. (38, 39) : 1.72 gm. of imidazole (Eastman Organic Chemicals, Rochester 3, New York) was dissolved in 90 ml. of 0.1 N HCl. The pH was carefully adjusted to 7.25 and the solution diluted to 100 ml with distilled water. This buffer (0.25 M) was stored in the refrigerator at 4°C.

D). Veronal Buffer

Sodium diethylbarbiturate 30.8 gm and barbituric acid 5.55 gm (Fisher Scientific Co.) were dissolved in 5 liters of demineralized water. The pH was carefully adjusted to 8.6 by addition of sodium diethylbarbiturate or barbituric acid and the volume was brought to 7.5 liters. This buffer (0.02 M) was usually prepared immediately before use.

E). Tricalcium Phosphate

This reagent was prepared according to Quick's method (40) : 31.6 gm of trisodium phosphate C.P. were dissolved in one liter of distilled water. In a separate container 13.3 gm of anhydrous calcium chloride C.P. were dissolved in one liter of distilled water. The trisodium phosphate solution was thoroughly stirred while the calcium chloride was slowly added to it. When the reaction was completed, the pH was adjusted

to 7 with dilute hydrochloric acid. The precipitated tricalcium phosphate was transferred to a one-liter jar and washed repeatedly with distilled water. Each day, the clear upper layer was tested for the presence of chloride with a silver nitrate solution. When all traces of chloride ions were removed, the volume of the tricalcium phosphate reagent was brought to four liters, thus making a 0.01 M suspension.

F). Pancreatic Trypsin Inhibitor of Kunitz and Northrop (Iniprol) (41).

This was a highly purified commercial preparation purchased from Choay Laboratories (Paris, France) and contained 200,000 anti-Schwert-Takenaka units (42) per milliliter.

G). Preparation of Fibrinogen

Fibrinogen was prepared from oxalated human plasma by double salt precipitation (43). The plasma was saturated with ammonium sulfate to 25% and the mixture stirred for 10 minutes. The suspension was then centrifuged at 10,000 rpm, at 0°C for 20 minutes and the supernatant discarded. The precipitate was dissolved in oxalated saline and dialyzed against the same solution under continuous stirring in order to remove all traces of sulfate ions; the dialysate was changed every hour for at least three times. In order to remove the prothrombin group of clotting factors (factors II, VII, IX, X) 15 ml of 0.1 M suspension of tricalcium phosphate was added to every 100 ml of the above fibrinogen solution and stirred for 10 minutes. The resulting mixture was centrifuged at 10,000 rpm for 20 minutes and the precipitate discarded. The supernatant was saturated with sodium chloride to 50% and stirred for 10 minutes. The fibrinogen thus precipitated was collected by centrifugation, redissolved in saline and dialyzed against saline until the dialysate became free of sulfate ions as tested by addition of barium chloride. The volume of the fibrinogen solution was measured and 0.2 M

sodium citrate equal to one-tenth the volume of the fibrinogen solution was added. The preparation was stored at -20°C if not used immediately. Storage did not exceed one week. Fibrinogen prepared by this method had a clottability of 95% and needed a shorter incubation time to become uncoagulable by thrombin than fibrinogen obtained by double precipitation with ammonium sulfate (43).

H). Preparation of AFIF (Anticoagulant Fraction of Incubated Fibrinogen)

Solutions (200 - 300 ml) containing 10 mg of fibrinogen per milliliter, 0.02 M sodium citrate, and 0.025 M, pH 7.3 imidazole buffer were sterilized by filtration through a Seitz filter and incubated under sterile conditions at 37°C . Each day an aliquot was taken and tested for coagulability by adding thrombin. As soon as an incubated solution became completely unclottable (end of clottable period (CP) or 100% CP (17)), one half of its volume was removed and 0.1 ml Kunitz's pancreatic trypsin inhibitor was added to every 100 ml of incubated fibrinogen to a final concentration of 200 anti-Schwert-Takenaka units (42, 44) per milliliter in order to stop the further action of fibrinolysin. The solution was then saturated with ammonium sulfate to 25% and the precipitate discarded. The supernatant was further saturated to 50% with ammonium sulfate and the precipitate was collected this time and dialyzed against saline to remove ammonium sulfate. The protein content of the fraction was determined by the biuret method of Kingsley, as described by Hawk et al. (45) and the preparation (AFIF of 100% CP) was stored at -20°C , if not used immediately.

The remainder of the fibrinogen solution was further incubated for an equal length of time. At the end of incubation, this incubated fibrinogen was fractionated as described above, and was designated as AFIF of 200% CP or overdigested AFIF.

I). Column Electrophoresis

This procedure was applied in order to separate the two main electrophoretic fractions of AFIF. A Porath electrophoresis apparatus model 3340 of L.K.B. Co. of Stockholm was used and Munktell's cellulose powder (Grycksbo Pappersbruk AB, Grycksbo, Sweden) was employed as a medium. The procedure was carried out in a cold room at 4°C, using 0.02 M veronal buffer of pH 8.6 (37). The concentration of each sample was adjusted to 50 - 60 mg protein per milliliter and the maximum volume of sample used was 5 ml. The protein, stained with bromphenol blue, was dialyzed against the veronal buffer with continuous stirring prior to electrophoresis. The current was applied at 20 ma and about 540 v until the blue-coloured band reached the bottom of the column (about 13 hours).

The flow rate of the eluting buffer was adjusted to one drop every 15 seconds and the fractions were collected at 20 minutes intervals. The protein concentration in each fraction was determined by reading the absorption at 280 mu in a Beckman DB spectrophotometer against a blank of veronal buffer at room temperature. The central fractions belonging to each peak were pooled together and shell frozen in dry ice and acetone. The frozen solutions were lyophilized until they became completely dry. The dried samples were then dissolved in a small quantity of saline and dialyzed against saline until free of veronal buffer (no absorption at 240 mu). The two main electrophoretic components obtained from overdigested AFIF were used in the prothrombin consumption test.

J). Preparation of Rabbit Brain Thromboplastin

The thromboplastin was prepared from fresh rabbit brain by Quick's acetone dehydration method (46). The cleaned (membranes and blood vessels removed) rabbit brains were put in a mortar, covered with 30 ml of acetone, and 0.1 ml

of 0.2 M sodium citrate was added to remove any traces of calcium. The material was crushed and mashed with a pestle. The spent acetone was poured off, 25 ml fresh acetone added and trituration repeated. This process was repeated several times until the material became granular and non-adhesive. The product was filtered and dried by suction. For storage 0.2 gm of this powder was transferred to 13 x 100 mm test tubes which were then covered with rubber stoppers, evacuated by means of a vacuum pump and stored at -20°C . For use 5 ml saline was added to a tube and the suspension was incubated at 50°C for 20 minutes. The tube was subsequently transferred to a water bath and kept at 37°C throughout the experiment.

K). Preparation of Bell-Alton Reagent

A brain extract, as a substitute for platelet suspension in the prothrombin consumption test, was prepared as described by Bell and Alton (47). Rabbit brain thromboplastin, obtained by acetone drying was further washed with acetone until free of cholesterol, as judged by a negative Liebermann-Burchard reaction. When 1 gm of the dried powder was extracted at room temperature with approximately 50 ml of chloroform, more than 300 mg of active material was obtained by evaporation of the filtrate. This gummy residue was finely homogenized in 50 ml of saline.

This preparation was further diluted 1:9 with saline before use. One volume of the diluted preparation was mixed with nine volumes native plasma (See "M") and the prothrombin consumption determined.

L). Preparation of Hemolysate

The hemolysate was prepared by the method of Quick, Georgatsos and Hussey (48). Nine volumes of blood were mixed with one volume of 0.1 M sodium oxalate and centrifuged at 1,000 rpm in an International Clinical Centrifuge

for 5 minutes. The supernatant platelet rich plasma was drawn off and replaced by an equal volume of physiological saline solution. The mixture was again centrifuged at 1,000 rpm for 5 minutes and the supernatant fluid removed and again replaced with fresh saline. The process was repeated three more times, but the speed of centrifugation was increased to 2,000 rpm and the time to 20 minutes. As much of the buffy layer as possible was removed. The packed red cells were resuspended in a volume of saline equal to that of the original blood and hemolyzed by freezing the suspension at -20°C and then rapidly thawing at 37°C . This preparation was used as a substitute of platelet factor 3 (49) in the performance of the prothrombin consumption test with platelet poor native plasma (See "M").

M). Preparation of Native Plasma

Blood collected with a siliconized syringe was centrifuged in precooled plastic tubes at 15,000 rpm for 15 minutes, at 4°C in a Servall refrigerated centrifuge, Model RC-2. The plasma was aspirated with a siliconized pipette, transferred into polyethylene tubes immersed in an ice bath, and used immediately in combination (10:1 or 9:1 respectively) with hemolysate or the Bell-Alton reagent; 0.6 ml of this mixture was used to replace 1 ml of whole blood in prothrombin consumption tests.

N). Preparation of Deprothrombinized Rabbit Plasma (50)

Rabbit blood was collected by heart puncture with a siliconized syringe and needle which had been precooled; nine volumes of blood were immediately mixed with one volume of 0.1 M sodium oxalate in a plastic centrifuge tube, the tube was centrifuged at 10,000 rpm for 5 minutes and the plasma withdrawn. To another plastic centrifuge tube 12.5 ml of 0.01 M tricalcium phosphate were added and centrifuged in a clinical centrifuge at top speed for 5 minutes

to pack the adsorbent. The water was poured off and the tube drained. Usually this was done before the blood was taken. To the packed tricalcium phosphate, 5 ml of fresh oxalated rabbit plasma was added and stirred for 5 minutes by means of a glass rod. The tube was then centrifuged at 15,000 rpm for 10 minutes. The plasma was carefully removed and kept in an ice bath. If deprothrombination was carried out properly, no clot would form when 0.1 ml of this plasma was mixed with 0.1 ml thromboplastin and 0.1 ml 0.02 M calcium chloride.

P). Determination of Prothrombin Time

Quick's one-stage procedure (51) was followed. Nine volumes of blood obtained by venipuncture with a silicone-coated syringe and needle were mixed with one volume of 0.1 M sodium oxalate and centrifuged at 3,000 rpm in an International Clinical Centrifuge for 10 minutes. One-tenth milliliter of the supernatant oxalated plasma was transferred to a standard test tube (13 x 100 mm) and 0.1 ml of rabbit brain thromboplastin preparation added. The tube was placed in a water bath (37°C) for a few seconds. Exactly 0.1 ml of 0.01 M calcium chloride was then blown forcibly into the tube and the stop-watch clicked at the same moment. The tube was placed in the water bath and gently agitated. A few seconds before the expected clotting time, the tube was removed from the bath and held above a magnifying mirror. The tube was observed from below and when the incipient web of fibrin appeared, the watch was stopped. Under these experimental conditions normal human plasma should have a prothrombin time of 12 seconds (51).

Q). Determination of Prothrombin Consumption

The method was essentially that described by Quick (52) to which an adsorption and elution step was introduced in order to eliminate AFIF, which

is not adsorbed on tricalcium phosphate, from the final step of prothrombin determination (13). Blood was obtained by venipuncture using a silicone-coated needle and syringe. The same volume of blood was transferred to three tubes which contained: a) AFIF dissolved in saline; b) plain saline (control of consumption;) c) oxalated saline (control of no consumption). The final concentration of AFIF varied from 1 mg/ml to 25 mg/ml according to the conditions of each experiment. Blood was mixed with the above mentioned solutions at the following ratios: a) 1:2 (33% blood), b) 2:3 (40% blood), c) 1:1 (50% blood) and d) 2:1 (66% blood) (Tables I, II, III, IV).

In order to prevent the action of any contaminating fibrinolysin, pancreatic trypsin inhibitor (Iniprol) was added to all mixtures including the controls to a final concentration of 1,000 anti-Schwert-Takenaka units per milliliter.

The various mixtures of blood were incubated in a water bath of 37°C . Fifteen minutes after the formation of a clot in the control mixture of blood and plain saline, all clots formed were gently loosened with wooden applicators and all the tubes irrespective of clot formation or not were centrifuged for 2 minutes in a clinical centrifuge at top speed in order to obtain the maximum production of plasma thromboplastin (52). The mixtures were further incubated for another 45 minutes and then centrifuged at 5,000 rpm in a refrigerated centrifuge (4°C) for 5 minutes. The serum (or plasma) was carefully aspirated. To the serum in the first and second tubes one-tenth of the original blood volume of 0.1 M sodium oxalate was added in order to facilitate the adsorptive action of tricalcium phosphate. To the third tube (oxalated plasma) saline was added instead.

The subsequent procedure closely followed Quick's adsorption and elution technique (50) with the exception that a double amount of tricalcium phosphate

TABLE I

Mixture of 33% Blood

(One volume of blood in three volumes of mixture)

Reagents added (ml)					
Iniprol	AFIF	0.1 M Na-oxalate	Saline	Blood	Total Volume (ml)
AFIF saline	0.03	4		2	6
Plain saline	0.03		4	2	6
Oxalated saline	0.03	0.2	3.8	2	6

Note: Final concentration of AFIF varied from 1 mg/ml to 10 mg/ml in the different experiments.

TABLE II

Mixture of 40% Blood

(Two volumes of blood in five volumes of mixture)

Reagents added (ml)					Total Volume (ml)
Iniprol	AFIF	0.1 M Na-oxalate	Saline	Blood	
AFIF saline	0.025	3		2	5
Plain saline	0.025		3	2	5
Oxalated saline	0.025	0.2	2.8	2	5

Note: Final concentration of AFIF was 15 mg/ml.

TABLE III

Mixture of 50% Blood

(One volume of blood in two volumes of mixture)

Reagents added (ml)					
Iniprol	AFIF	0.1 M Na-oxalate	Saline	Blood	Total Volume (ml)
AFIF saline	0.03	3		3	6
Plain saline	0.03		3	3	6
Oxalated saline	0.03	0.3	2.7	3	6

Note: Final concentration of AFIF varied from 10 mg/ml to 25 mg/ml in the different experiments.

TABLE IV

Mixture of 66% Blood

(Two volumes of blood in three volumes of mixture)

Reagents added (ml)					
Iniprol	AFIF	0.1 M Na-oxalate	Saline	Blood	Total Volume (ml)
AFIF saline	0.03	2		4	6
Plain saline	0.03		2	4	6
Oxalated saline	0.03	0.4	1.6	4	6

Note: Final concentration of AFIF was 15 mg/ml.

suspension were transferred to a plastic tube and packed as described in "N". To the packed tricalcium phosphate, 2 ml of the oxalated serum (or plasma) to be analyzed was added and mixed with a glass rod for 5 minutes. The tubes were centrifuged at 15,000 rpm for 10 minutes, the clear plasma was poured off and the tubes drained. The adsorbent was washed by adding 5 ml saline solution and stirring with a glass rod. The tricalcium phosphate was again packed by centrifugation as before, the saline was removed by decanting, and the sides of the tubes were dried with adsorbent tissue. Sodium citrate (0.2 M), at one-tenth the volume of adsorbed plasma or serum, was thoroughly mixed with the packed adsorbent for 5 minutes. The suspension was then centrifuged at 15,000 rpm for 10 minutes and the clear supernatant eluate transferred to a clean tube. Of this eluate 0.1 ml was mixed with 0.9 ml deprothrombinized rabbit plasma to yield "reconstituted plasma". A mixture of 0.1 ml calcium chloride (0.2 M) and 0.1 ml thromboplastin was placed in a water bath at 37°C for several seconds. Into this mixture 0.1 ml reconstituted plasma was forcibly blown and the clotting time carefully determined with the aid of a magnifying mirror. Thus the quantitative determination of the prothrombin remaining in the serum, after the blood has coagulated under standardized conditions, using the one-stage prothrombin time, constitutes the prothrombin consumption test (52). Increasing degrees of prothrombin consumption in the test fluid is indicated by increasing values in seconds. Quick (52) has suggested that the prothrombin consumption test measures factors that produce available thromboplastin.

III RESULTS

A). Effect of Dilution, Incubation, Adsorption and Elution on the One-stage Prothrombin Time.

Since the blood used to determine the effect of AFIF on prothrombin consumption was first diluted and incubated at 37°C before any measurement of prothrombin activity could be made, it was necessary to consider the effect of dilution and incubation on the one-stage prothrombin time. In addition AFIF had to be eliminated from the actual measurement of prothrombin activity, since it inhibits the action of thrombin and consequently it could prolong the thrombin time independently of any effect on prothrombin consumption. Prothrombin was therefore removed from the blood mixtures by adsorption on tricalcium phosphate and the test was performed on the eluate. For this reason the effect of adsorption and elution (50) on the prothrombin time was reinvestigated. Fresh human blood was mixed 9:1 (90% blood), 1:1 (50% blood) and 1:2 (33% blood) with saline to which 0.1 M sodium oxalate had been added at a volume equal to one tenth the volume of blood. The plasma was separated by centrifugation and the one stage prothrombin time was determined (51) immediately. The remainder of the plasmas were then incubated in a water bath at 37°C. The tubes were covered with parafilm in order to prevent changes in pH during incubation (14). After one hour the prothrombin of the plasmas was again determined by: a) using each plasma alone and b) mixing an aliquot of each plasma (9:1) with fresh deprothrombinized rabbit plasma (50). In other experiments 2 ml of each incubated plasma were

TABLE V

Effect of Dilution, Incubation, Adsorption and Elution on the
One-Stage Prothrombin Time

Concentration of Blood		Prothrombin Time (sec.)			
		Fresh Test Plasma	After one hour incubation		
			Test Plasma	Test Plasma + Rabbit Plasma*	Adsorption & Elution
90% (9:1)	1	12	14	9	9
	2	12	14	9.5	9
	3	12	14	10	10
50% (1:1)	1	17	20		13.5
	2	16	20	13.5	12
33% (1:2)	1	20	20	14	13.5
	2	20	21	15	15

* Nine parts of test plasma mixed with one part of fresh deprothrombinized rabbit plasma.

transferred to a test tube containing packed tricalcium phosphate. The prothrombin was adsorbed on the latter and subsequently eluted with 0.2 M sodium citrate as described in Materials and Methods. The prothrombin time was again determined after mixing the eluate with deprothrombinized rabbit plasma.

Table V shows that the prothrombin time is prolonged by dilution. The values obtained are similar to those of Quick (51), but not identical, probably since the dilutions used in each case were different. Incubation similarly prolonged the prothrombin time at blood concentrations of 90% and 50% but it had no significant effect in the 33% blood mixtures. Addition of fresh deprothrombinized rabbit plasma overcorrected the effect of incubation and corrected to a considerable extent the effect of dilution. The use of the adsorption and elution technique did not essentially change the effect of deprothrombinized rabbit plasma. The slightly prolonged prothrombin time of some mixtures of incubated plasma with rabbit plasma in comparison to the values obtained by the adsorption and elution technique was probably due to the diluting effect of the rabbit plasma which was added to these mixtures.

B). Stability of the Inhibitory effect of AFIF on Prothrombin Consumption with respect to the Incubation Stage of the Parent Fibrinogen Solution. Effect of Anticoagulant and Blood Concentration.

Early work has demonstrated that human AFIF inhibits the consumption of prothrombin in shed blood (13). It is well established that the antithrombic activity of AFIF varies with the stage of incubation of the parent fibrinogen solution (13, 17, 23). Samples isolated when the parent fibrinogen solution becomes unclottable by thrombin i.e. at the end of the clottable period (100% CP) show the strongest antithrombic effect. When the incubation is allowed to continue further for an equal length of time (200% CP), the

anticoagulant which is then obtained exhibits an appreciably weaker antithrombic activity. It was considered warranted therefore to investigate whether the ability of AFIF to prevent the consumption of prothrombin is similarly affected by the length of incubation of the parent fibrinogen solution.

Preparations of AFIF isolated from incubated human fibrinogen at 100% and 200% CP were mixed with blood obtained from healthy human volunteers, in ratios 1:1, 1:2, 2:3 and 2:1. The final concentration of AFIF varied from 1-25 mg/ml. Aliquots from each blood were mixed with saline or oxalated saline at the same ratios as with AFIF and these served as controls. Prothrombin consumption was determined as described in Materials and Methods.

In Table VI, experiments 1, 2 and 3, using 100% CP AFIF were paired with experiments 1, 2 and 3 respectively, using 200% CP AFIF i.e. the respective 100% and 200% CP AFIF preparations were obtained from the same incubated fibrinogen solution, the blood aliquots to which these were added were withdrawn from the same individual. Throughout the various final concentration of AFIF (1, 2.5, 5 and 10 mg/ml) in each of these experiments, blood from the same donor was used except that the blood for experiments 1 of 100% CP and 200% CP, at the concentration of 10 mg/ml of AFIF, came from a separate donor.

Because of the amount of work involved to carry out simultaneously such a large number of determinations, the remaining experiments (Tables VII and VIII) were performed on blood samples from different donors. As in the paired experiments, however, the respective dilutions were made with the same AFIF preparations.

TABLE VI

Effect of AFIF Concentration on Prothrombin Consumption
of Blood Diluted to 33%

		Prothrombin Consumption (sec.)			
Final Concentration of AFIF (mg/ml)	Diluent	1	2.5	5	10
		AFIF Sal*Ox-Sal**	AFIF Sal Ox-Sal	AFIF Sal Ox-Sal	AFIF Sal Ox-Sal
AFIF isolated at 100% CP***	1	43 45 15	22 45 15	16 45 15	11 42 16
	2	17 25 15	12.5 25 15	12.5 25 15	12 25 15
	3	21 48 14.5	13 48 14.5	13 48 14.5	11 48 14.5
200% CP	1	30 45 15	22 45 15	12 45 15	12 42 16
	2	23 25 15	11.5 25 15	11.5 25 15	11.5 25 15
	3	14 48 14.5	11.5 48 14.5	11.5 48 14.5	11.5 48 14.5

* Sal = Saline

** Ox-Sal = Oxalated Saline

*** CP = Clottable period = initial part of incubation (fibrinogenolysis) during which the parent fibrinogen solution was still clottable by thrombin (17).

TABLE VII

Effect of AFIF Concentration on Prothrombin Consumption
of Blood Diluted to 50%

Prothrombin Consumption (sec.)

Final Concentration of AFIF (mg/ml) Diluent	10		15		20		25	
	AFIF	Sal*Ox-Sal**	AFIF	Sal	AFIF	Sal	AFIF	Sal
AFIF isolated at 100% CP***	1	19.5	51	16.5	11.5	51	16.5	
	2			40	14	40	12.5	12.5
	3			41	15.5	33	12.4	12.4
200% CP	1	47	48	12.5	14	48	12.5	12.5
	2			55	11.5	55	13.5	13.5
	3			44	20.5	33	12.4	12.4

* Sal = Saline

** Ox-Sal = Oxalated Saline

*** CP = Clottable period = initial part of incubation (fibrinogenolysis) during which the parent fibrinogen solution was still clottable by thrombin (17).

TABLE VIII

Effect of Blood Concentration on Prothrombin Consumption at a

Constant Concentration of AFIF

(15 mg/ml final mixture)

Prothrombin Consumption (sec.)

Blood Concentration (per cent)	33		40		50		66
Diluent	AFIF	Sal*Ox-Sal**	AFIF	Sal Ox-Sal	AFIF	Sal Ox-Sal	AFIF Sal Ox-Sal
AFIF isolated at							
1	10.5	42 16	25	42 14	40	41 13	38 36 11.5
2			12	60 16.5	16.5	59 15.5	37 58 13.2
3			8.5	44 14.5	30	44 40	40 44 12.5
200% CP							
1	9.3	30 14	7	33.5 11.5	28	36.5 11.5	55 49 14.5
2	10.2	42 16	10	43 13.5	36	43.5 12	55 43 12
3			11.5	50 14	27	55.5 13.5	33 56 12.5

* Sal = Saline

** Ox-Sal = Oxalated Saline

*** CP = Clottable period = initial part of incubation (fibrinogenolysis) during which the parent fibrinogen solution was still clottable by thrombin (17).

Table VI shows that there is no meaningful difference between AFIF preparations isolated at 100% and those at 200% CP, concerning the degree of inhibition of prothrombin consumption. This observation also holds for higher concentration of AFIF used with less diluted blood shown in Table VII. As seen from Table VIII the extent of inhibition depends also on blood concentration. With both kinds of preparations then, the inhibition depends upon: a) the concentration of the anticoagulant, b) the dilution of the blood (the greater the concentration of the anticoagulant or the dilution of the blood, the greater the inhibition. In favourable conditions of blood dilution and/or anticoagulant concentration there is an indication that prothrombin times shorter than those obtained in the presence of oxalate were observed).

C). Effect of the two main Electrophoretic Fractions of AFIF on Prothrombin Consumption.

Using column electrophoresis, overdigested AFIF i.e. preparations isolated at twice the time required for the parent fibrinogen solution to become unclottable (at 200% CP), were separated into the two main fractions: the large peak (α -derivative of Seegers' et al. (7)) and the small peak (β -derivative of the same authors, the more electronegative), as shown in Figure 1. Due to the fact that only a small quantity of protein could be obtained each time, especially from the small peak component, experiments were carried out using only low concentrations of blood (33%); and peaks obtained from two or three runs were combined together.

Table IX shows that both fractions are able to inhibit prothrombin consumption, although higher concentrations than that of the original AFIF

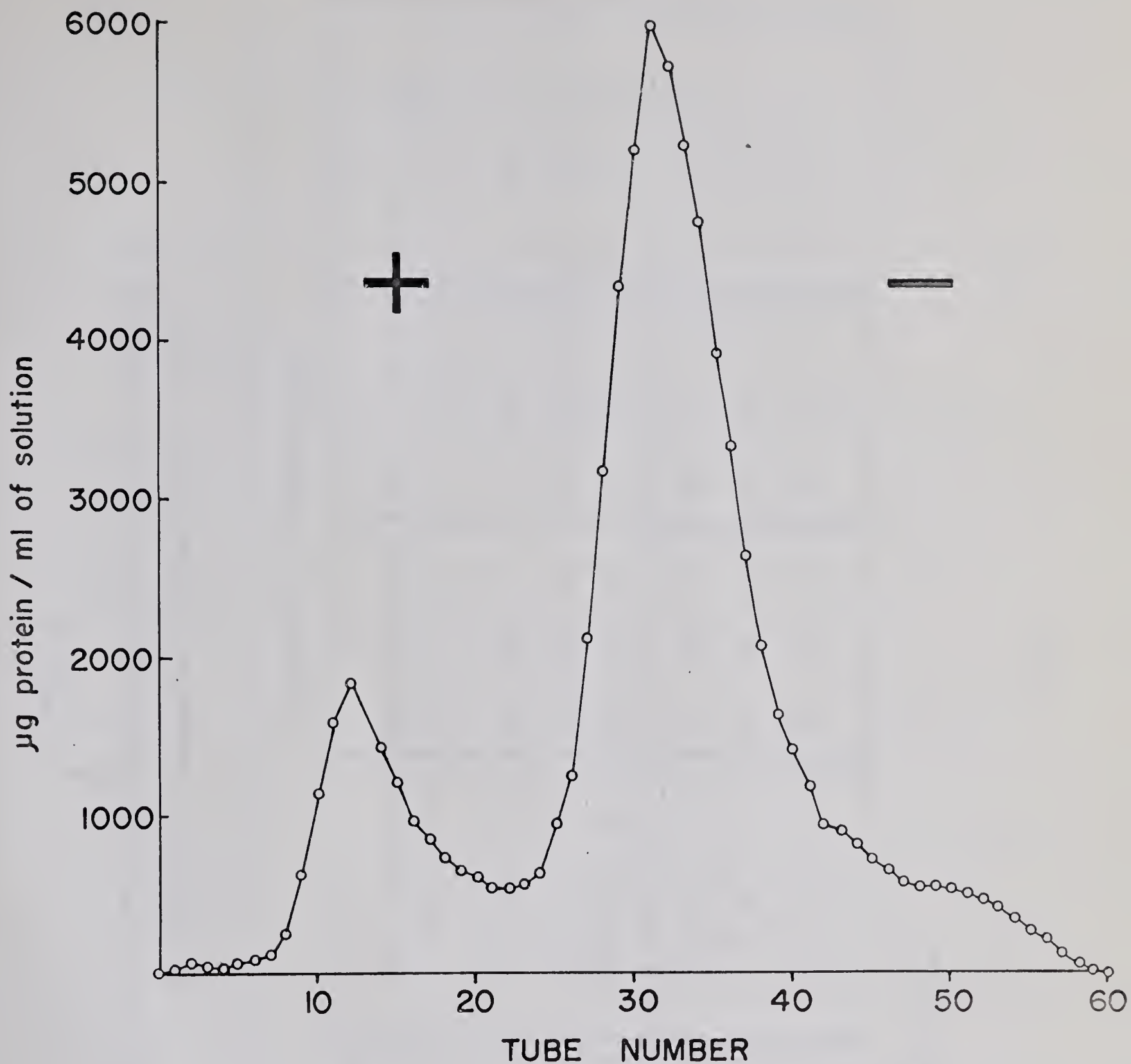


Fig. I.

Electrophoretic mobility of human AFIF of 200% CP
(Column electrophoresis in Veronal buffer 0.02 M,
pH 8.6 , 20 ma , \approx 540 V.)

TABLE IX

Effect of the Two Main Electrophoretic Fractions of AFIF (200% CP)
on Prothrombin Consumption in Blood Diluted to 33%

Prothrombin Consumption (sec.)													
Final Concentration of AFIF (mg/ml) Diluent		1		2.5		5		15					
		AFIF Sal*Ox-Sal**		AFIF Sal Ox-Sal		AFIF Sal Ox-Sal		AFIF Sal Ox-Sal					
Fractions of AFIF													
Large peak	1	75	60	17	81	60	17	53	60	17	9	32	15
	2	26	20	13	37	20	17	26	20	17	17	32	15
	3	60	45	18	65	45	18	27	45	18	15	45	18
Small peak	1	48	43	17	52	43	17	40	43	17	10.5	43	15
	2	38	32	15	46	32	15	39	32	15	9	45	14
	3	45	32	15	44	32	15	21	32	15	8.5	45	14

* Sal = Saline

** Ox-Sal = Oxalated Saline

(Table VI) are required in order to have a complete inhibition.

D). Effect of Hemolysate and the Bell-Alton Reagent on the Inhibitory Activity of AFIF on Prothrombin Consumption.

It has been observed (Triantaphyllopoulos, D.C., unpublished results) that platelets in blood rendered uncoagulable by AFIF do not undergo viscous metamorphosis. The possibility therefore was investigated whether the anti-prothrombin - consumption activity of AFIF is due to the prevention of platelet disintegration.

Platelets were removed from native plasmas by centrifugation and hemolysate or diluted (1 : 9) Bell-Alton reagent (crude cephalin) was added as a platelet substitute. Aliquots of these mixtures were added 0.6 : 1 to solutions of AFIF (final concentration : 30 mg/ml), saline or oxalated saline and the prothrombin consumption was determined.

The results in Tables X and XI show that the anti-prothrombin - consumption activity of AFIF is lost when the platelets are substituted by hemolysate or the Bell-Alton reagent. It is worth noting that in the presence of the last reagent AFIF not only does not prevent the consumption of prothrombin, but, on the contrary, it seems to facilitate it. This facilitation is remarkable in view of the fact that the Bell-Alton reagent alone, at the concentrations used in these experiments, exerts an inhibitory effect on prothrombin consumption.

The effect of the Bell-Alton reagent on prothrombin consumption is not affected by the presence of platelets, as shown in Table XII.

E). Effect of Pancreatic Trypsin Inhibitor on Prothrombin Consumption.

It has been reported that pancreatic trypsin inhibitor does not affect blood coagulation (44). However, specific tests of the effect of this

TABLE X

Effect of Hemolysate on the Anti-prothrombin-consumption

Activity of AFIF in Native Plasma

		Prothrombin Consumption (sec.)			
		AFIF	Saline	Oxalated	Saline
AFIF isolated at					
100% CP	1	89	81		14
	2	80	81		14
	3	90+	54		14
	4	90+	54		14
	5	87	65		13.5
200% CP	1	79	81		14
	2	54	81		14
	3	90+	54		14
	4	84	65		13.5
	5	70	65		13.5

Experimental conditions: 1.62 ml platelet - poor native plasma + 0.17 ml hemolysate + 2.93 ml AFIF, saline or oxalated saline. Final concentration of AFIF 30 mg/ml.

TABLE XI

Effect of Bell-Alton Reagent on the Anti-prothrombin-consumption

Activity of AFIF in Native Plasma

		Prothrombin Consumption (sec.)		
		AFIF	Saline	Oxalated Saline
AFIF isolated at				
100% CP	1	90	15	14.5
	2	95	15	14.5
	3	70	14.2	13.5
	4	80	14.2	13.5
	5	72	38	13
200% CP	1	77	15	14.5
	2	81	15	14.5
	3	72	14.2	13.5
	4	60	38	13

Experimental conditions: 1.62 ml platelet poor native plasma + 0.18 ml diluted (1:10) Bell-Alton reagent + 2.92 ml AFIF, saline or oxalated saline. Final concentration of AFIF 30 mg/ml.

TABLE XII

Effect of Bell-Alton Reagent on the Anti-prothrombin-consumption

Activity of AFIF in Whole Blood

		Prothrombin Consumption (sec.)		
		AFIF	Saline	Oxalated saline
AFIF isolated at				
100% CP	1	27	8	12
	2	44	8.5	13.5
	3	47	8.5	13.5
	4	34	8	13

Experimental conditions: 3 ml blood + 0.18 ml diluted (1:9)
 Bell-Alton reagent + 2.92 ml AFIF, saline or oxalated saline.
 Final concentration of AFIF 30 mg/ml.

TABLE XIII

Effect of Pancreatic Trypsin Inhibitor (Iniprol) on Prothrombin
Consumption of Blood Diluted to 33%

Experiments	Prothrombin Consumption (sec.)		
	Iniprolized Saline*	Saline	Oxalated Saline
1	57	57	14
2	23	35	14
3	13	24	11
4	15	32	15
5	16	28	11

* Final concentration of Iniprol: 1333 anti-Schwert-Takenaka units (42) per milliliter.

TABLE XIV

Effect of Pancreatic Trypsin Inhibitor (Iniprol) on Prothrombin
Consumption of Blood Diluted to 40%

Experiments	Prothrombin Consumption (sec.)		
	Iniprolized Saline*	Saline	Oxalated Saline
1	61	64	15.4
2	45	38	17
3	40	50	10.5
4	24	32	12

* Final concentration of Iniprol: 1200 anti-Schwert-Takenaka units (42) per milliliter.

TABLE XV

Effect of Pancreatic Trypsin Inhibitor (Iniprol) on Prothrombin
Consumption of Blood Diluted to 50%

Experiments	Prothrombin Consumption (sec.)		
	Iniprolized Saline*	Saline	Oxalated Saline
1	61	54	16
2	40	38	11.5
3	36	37	13.7
4	35	28	11
5	26	26	11

* Final concentration of Iniprol: 1000 anti-Schwert-Takenaka units (42) per milliliter.



Figure 2. Effect of pancreatic trypsin inhibitor on clot retraction. The first, second and third (starting from left) tubes contained blood diluted to 33% and the fourth and fifth blood diluted to 50%. To the second, third and fifth tubes pancreatic trypsin inhibitor was added to a final concentration of 1000, 1333 and 1000 anti-Schwert-Takenaka units per milliliter, respectively. Picture was taken one hour after blood was drawn.

inhibitor on prothrombin consumption were not performed. Since this inhibitor was used in all experiments, it was considered necessary to examine whether or not it had any effect on prothrombin consumption.

Blood was mixed 1 : 2 (33%), 1 : 1.5 (40%) and 1 : 1 (50%) with plain saline, oxalated saline and saline containing 2000 anti-Schwert-Takenaka units of pancreatic trypsin inhibitor (Iniprol) per ml (= iniprolized saline). As Tables XIV and XV indicate Iniprol has no effect on prothrombin consumption, when the concentration of blood is 40 or 50 per cent. However, when the blood concentration is 33% inhibition is observed (Table XIII).

It was noticed incidentally that in the presence of Iniprol clot retraction is retarded (Figure 2).

IV DISCUSSION

The present study confirms the observation made by Quick (51) that dilution of blood (or plasma) with saline prolongs the one-stage prothrombin time. He postulated (51) that if thromboplastin and calcium were held at an optimal concentration, the rate of thrombin formation as measured by the clotting time is a function of the concentration of prothrombin.

Short incubation increased the prothrombin time of blood (Table V). Quick (53) has shown that storage does the same thing and he attributed this phenomenon to the loss of factor V (labile factor, Ac-globulin). It is interesting to find that the substance(s) responsible for the destruction of factor V during incubation is (are) inactivated by dilution (Table V). This finding supports the concept of Tocantins (54) and Seegers (55) that blood coagulation is influenced by many forces (anticoagulants and pro-coagulants) in equilibrium and that this equilibrium changes with dilution. Addition of fresh deprothrombinized rabbit plasma to the plasma obtained from one-hour incubated blood shortened the prothrombin time of the latter. This finding is similar to Quick's (53) observation with stored plasma. Quick showed that the shortening action of rabbit plasma is due to its large content in factor V. Rapaport, Aas and Owren (56) believe that activation of factor VII contributes to this effect. The results obtained with the adsorption and elution technique (Table V) support Quick's claim that this procedure does not change the one-stage prothrombin time (50).

The results of this study show that the inhibitory effect of AFIF

on prothrombin consumption (13) cannot be due to the action of contaminating fibrinolysin. Pancreatic trypsin inhibitor (Iniprol) was added to all mixtures at a concentration sufficient to inhibit all the potential fibrinolytic activity of blood (44). Neither can this antiprothrombin - consumption activity of AFIF be due to "Iniprol". All the saline controls in these experiments contained Iniprol and all had good prothrombin consumption. Using the two-stage method (57) Triantaphyllopoulos (58) confirmed the results obtained by the one-stage assay.

As mentioned earlier, the antithrombic activity of AFIF depends greatly on the incubation stage of the parent fibrinogen solution (13, 17, 18, 23). This activity is low at the beginning of incubation but increases steadily reaching a peak when the fibrinogen solution becomes completely unclottable (i.e. at the end of the clottable period) and decreases to a low plateau during advanced stages of incubation. Thus preparations of AFIF isolated at 100% CP exert a considerably higher antithrombic effect than samples obtained at 200% CP. Tables VI, VII and VIII show that, unlike the antithrombic activity, the anti-prothrombin-consumption ability of AFIF is unaffected by the length of incubation. Preparations from 200% CP can inhibit consumption to the same extent as those from 100% CP. The inhibition of consumption depends only on the final concentration of the blood and the anticoagulant used. Since the number of platelets and the amount of factor VIII (antihemophilic globulin) fluctuate widely from individual to individual, it is difficult to obtain a constant figure. Only those results which came from experiments using the same blood sample can be easily compared. However, Table VI, Table VII and Table VIII do show qualitatively a change of AFIF anti-prothrombin-consumption activity. Approximately 20 mg/ml,

15 mg/ml, and 5 mg/ml (final concentration of AFIF) can completely inhibit prothrombin consumption in all cases in 50%, 40% and 33% blood respectively, It should be noticed that in some instances the prothrombin time of the AFIF sample was shorter than the respective oxalated control. On the basis of the available experimental data, the significance of this observation is in doubt and no explanation can be given for this phenomenon at the present time.

The experiments with the two main electrophoretic fractions of AFIF indicate that both of them interfere with normal consumption (Table IX). This probably can explain why AFIF preparation isolated at different stages of incubation (100% and 200% CP) have the same activity. The fact that a higher concentration of electrophoretic components than that of the original AFIF was required in order to inhibit prothrombin consumption completely indicates that some of the anti-prothrombin-consumption activity is lost during the process of separation.

The results in Table X and Table XI show that the anti-prothrombin-consumption activity of AFIF is lost when platelets are substituted by hemolysate or the Bell-Alton reagent (rabbit brain phospholipid). These experiments suggest that AFIF may exert its activity by means of an anti-viscous metamorphosis effect. Since it is known that thrombin is indispensable for the viscous metamorphosis of platelets and since AFIF is an antithrombin, it is likely that the above mentioned effect of AFIF is an indirect one. However, additional mechanism(s) of action is(are) suggested by the fact that in some cases, AFIF still inhibited the prothrombin consumption although the blood had clotted, in other words in a condition in which it is reasonable to assume that viscous metamorphosis had already occurred.

It is worth noting that in the presence of the Bell-Alton reagent (crude cephalin) AFIF not only does not prevent the consumption of prothrombin

but on the contrary, it seems to facilitate it. This facilitation should not be considered as an action of the Bell-Alton reagent itself because this reagent alone (at a dilution of 1:100 of the original preparation) inhibits prothrombin consumption. Experiments using the whole blood instead of native plasma showed that these properties are not affected by the presence of platelets (Table XII). Formation of a new substance which accelerates the prothrombin consumption may be possible. Recently, Shaw (59) found that high concentrations of platelets can remove factor IX from the blood and therefore reduce thromboplastin generation. It is possible that the Bell-Alton reagent may act in an analogous way, i.e. remove a factor(s) which is (are) essential for the formation of plasma thromboplastin. The latter is known (52) to determine the consumption of prothrombin. Addition of AFIF may counteract this action. We have no direct evidence of the above two speculations. Further investigation on this subject is necessary.

In the last experiments, it was found (Tables XIV, XV) that pancreatic trypsin inhibitor had no effect on prothrombin consumption when the concentration of blood was high. However, when the blood was very diluted (33%) and a slightly higher concentration of Iniprol (1,333 units/ml) was used, an inhibition did occur in some cases (Table XIII). It was noticed in addition that in the presence of Iniprol the retraction of clots was retarded (Figure 2). These findings cannot rule out the existence of an anti-prothrombin-consumption property of AFIF. The reasons are twofold: (1) this phenomenon does not appear in the experiments of 40% and 50% blood where AFIF shows anti-prothrombin consumption activity, and (2) as mentioned earlier all saline controls (Tables VI, VII, VIII), which contained pancreatic trypsin inhibitor had very high prothrombin consumption. It is

possible that the pancreatic trypsin inhibitor may simply retard the consumption of prothrombin without permanently inhibiting it. From the fact that the pancreatic trypsin inhibitor retards clot retraction, one may conclude that this inhibitor interferes with the formation of thrombin from prothrombin, since thrombin is necessary for the contraction of thrombosthenin (60, 61, 62).

V SUMMARY

Previous work had shown that fibrinogen acquires anti-coagulant properties upon incubation; that the anticoagulant fraction of incubated fibrinogen (AFIF) inhibits the clotting of fibrinogen by thrombin in a competitive manner; that AFIF interferes with both the hydrolytic action of thrombin on fibrinogen and the polymerization of the fibrin monomers; and that AFIF also inhibits the consumption of prothrombin.

The present study shows that:

1. The inhibitory effect of AFIF on prothrombin consumption is not due to contaminating fibrinolysin but an intrinsic property of AFIF itself. This property is unaffected by the length of incubation of the parent fibrinogen solution. Both 100% and 200% CP AFIF possess an equal strength of inhibition.
2. The degree of inhibition is affected by the final concentration of blood and anti-coagulant used. Approximately 20 mg/ml, 15 mg/ml, and 5 mg/ml (final concentration) of AFIF can completely inhibit prothrombin consumption in all cases in 50%, 40% and 33% blood respectively.
3. Both main electrophoretic components of AFIF were found to be responsible for the inhibition.
4. AFIF lost its inhibitory activity on prothrombin consumption when platelets were substituted by red cell hemolysates or rabbit brain phospholipid (Bell-Alton reagent). This seems to indicate that blocking of viscous metamorphosis of platelets is a major action of AFIF

in its inhibition on prothrombin consumption. The Bell-Alton reagent acted as an anti-coagulant and AFIF as a procoagulant under these experimental conditions.

5. Kunitz's pancreatic trypsin inhibitor (Iniprol) does not affect blood coagulation when blood concentration is 40% or higher. However, when blood is 33% Iniprol prolongs blood coagulation time and retards clot retraction.

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